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Molecular Identification of Arbuscular Mycorrhizal Fungal Spores Collected in Korea

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Arbuscular mycorrhizas (AM) have mutualistic symbiosis with plants and thus efforts have been placed on application of these symbiotic relationships to agricultural and environmental fields. In this study, AM fungi were collected from 25 sites growing with 16 host plant species in Korea and cultured with *Sorghum bicolor* in greenhouse condition. AM fungal spores were extracted and identified using both morphological and molecular methods. Using morphological characters, total 15 morpho-speices were identified. DNA was extracted from single spore of AM fungi and a partial region on 18S rDNA was amplified using nested PCR with AM fungal specific primers AML1/AML2. A total of 36 18S rDNA sequences were analyzed for phylogenetic analysis and 15 groups of AM fungi were identified using both morphological and molecular data of spores. Among the species, 4 species, *Archaeospora leptoticha, Scutellospora castanea, S. cerradensis, S. weresubiae* were described for the first time in Korea and two species in *Glomus* and a species in *Gigaspora* were not identified. Morphological and molecular identification of AM fungal spores in this study would help identify AM fungal community colonizing roots.

KEYWORDS: Arbuscular mycorrhiza, 18S rDNA, Specific primer

Arbuscular mycorrhizal (AM) fungi have mutualistic symbiosis with most vascular plants and provide many benefits to the plants including increased uptake of inorganic nutrients, enhanced tolerance to many environmental stress and protection from pathogens (Allen, 1991; Smith and Read, 1997). Also, species diversity of AM fungi influences plant diversity and ecosystem productivity (van der Heijden *et al.*, 1998). Thus efforts have been placed on application of these symbiotic relationships to agricultural and environmental fields. In spite of these advantages, application of these fungi has been limited because their relationships with plants have not been clearly understood. It would be mainly due to the inability to culture these fungi because they are obligate symbionts which can only be cultured under the presence of their hosts.

Identification of AM fungi have been relied on microscopic observations of spores collected from soil (Bethenfalvay and Yoder, 1981; Schenck and Perez, 1990). About 200 different AM fungal taxa in order Glomales of Zygomycota have been described on the basis of morphological characteristics of their asexual spores (Morton and Benny, 1990). However, these fungal organs have a limited number of morphological features. In recent years, molecular techniques have been used to study phylogenetic relationships and genetic variations of AM fungi. Several attempts have made to use ITS region of rDNA as a tool for identification of AM fungi (Redecker *et al.*, 1997) and phylogenetic studies with DNA extractions and sequencing from AM fungal spores (Redecker *et al.*, 2000; Morton and Redecker, 2001). This fungal group has recently elevated to the status of a new monophyletic phylum Glomeromycota (Schuessler *et al.*, 2001). This is based on the analysis of small subunit rDNA sequences.

Spores are formed in soil during certain period of their life cycle and related to their host plants and environmental stress. Also, some species of AM fungi may not produce their spores. The contrary to spores in soil, hyphae colonizing the root are an active part of the fungus involved in interconnections between plant and soil environment. However, hyphae in roots can not be identified to species level with morphological methods. Thus, it is important to develop techniques identifying AM fungi colonizing roots for further studies and applications of these fungi. The molecular technique allows identifying hyphae within roots with AM specific PCR primers targeted rDNA regions. The specificity of primers is an important factor for molecular identification of AM fungi within roots. Recently, AM specific primer set AML1/AML2 has been developed. These primers amplified most of the fungi belonging to Glomeromycota and excluded DNA of other organisms such as plant, bacteria and other fungi inhabiting roots, suggesting high specificity of the primers. Also, because molecular identification of AM fungal hyphae colonizing roots depends on molecular information of spores, it is important to obtain morphological and molecular data of spore for molecular identification of the fungi in roots.

About 50 species of AM fungi have been reported in

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Korea (Lee *et al.*, 2003) and their identification was based on morphological characteristics of mostly field collected spores. There is little information on nucleotide sequences of AM fungal spores found in Korea. In this study, to obtain morphological and molecular data from AM fungal spores, spores were isolated from cultures with soils collected from various sites in Korea and identified using morphological characteristics. Also, a partial 18S rDNA gene was amplified from single spore and molecular identification was performed by the sequence analysis of the gene.

Materials and Methods

Soil collection and culture of AM fungal spores. Soil samples were collected from the areas surrounding 22 different host species in 30 sites of Korea (Fig. 1). Plants were identified on the basis of Illustrated Flora of Korea (Lee, 1985). The collected soils were mixed with sterilized sand at the rate of 1 : 1 (w/w) and were used for trap cultures for AM fungi. Pots (15 cm diam.) for trap culture were maintained with *Sorghum bicolor* as host plants under greenhouse condition for four months. The pots were watered as needed and fertilized every 2 weeks with 100 *ml* of low P (1/20 phosphate) Hoagland Nutrient Solution (2.8 g H_3BO_3, 3.4 g $MnSO_4 \cdot H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 16.22 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_6MO_7O_2 \cdot 4H_2O$, 5 *ml* H_2SO_4, 6.72 g Na_2EDTA, 5.58 g $FeSO_4$, 0.94 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.66 g KNO_3, 0.06 g $HN_4H_2PO_4$). After 4 months of growth, aboveground parts of plants were removed and soils in the pots were stored at 4°C until used.

Morphological identification of AM fungal spores. Spore of AM fungi were extracted from 10 g of soil using wet-sieving and sucrose density gradient centrifugation methods (Daniels and Skipper, 1982). The extracted spore was observed under a light microscope and identified morphologically based on spore color, shape, surface ornamentation, spore contents and wall structures (Bethenfalvay and Yoder, 1981; Schenck and Perez, 1990).

Molecular Identification of AM fungal spores. Single spores of morphologically identified spores were separated and used for molecular identification. The spore was washed with distilled water three times, crushed to extract DNA in a 0.2 *ml* PCR tube and sterilized water 1 μl added to a 0.2 *ml* PCR tube. Partial 18S rDNA fragments of AM fungi were amplified by nested PCR (van Tuinen *et al.*, 1998). The first PCR was performed using AM fungal specific primer AML1 (5'-AAC TTT CGA TGG TAG GAT AGA-3') and universal primer NS4 (5'-TTC CAT CAA TTC CTT TAA G-3') for 30 cycles (1 cycle at 95°C for 3 min, at 45°C for 1 min, at 72°C for 1min 30 sec; 28 cycles at 95°C for 30 sec, at 45°C for 1 min at 72°C for 1 min 30 sec; 1 cycle at 95°C 30 sec, at 45°C for 1 min, at 72°C for 10 min). DNA (about 900 bp) amplified by PCR was separated on 1% agarose gel, stained with ethidium bromide (EtBr) and checked under UV trans-illuminator. The first PCR product was used as template for the second amplification with AM fungal specific primers, AML1 and AML2 (5'-CCA AAC ACT

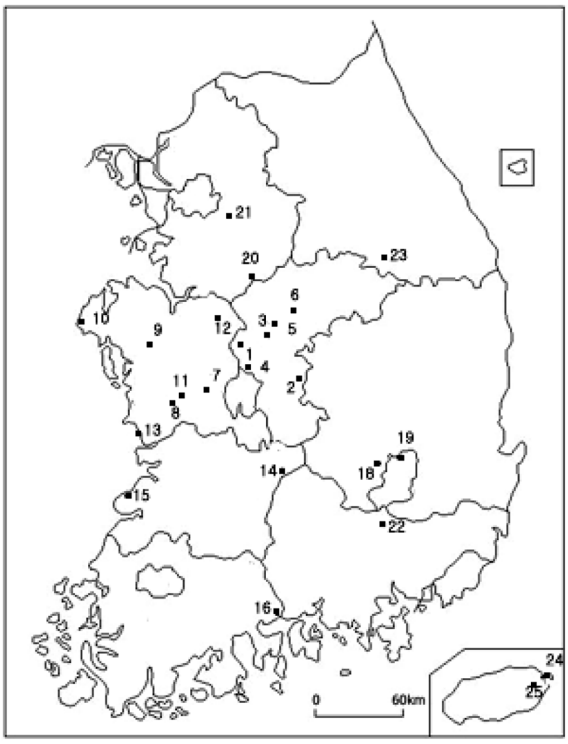

Fig. 1. Sites of soil samples for arbuscular mycorrhizal fungal spores and spore isolate numbers in the parenthesis used in this study. 1. Korea National University of Education, Chung-buk (CB), (CB01006-1); 2. Mt. Gubyeong, CB, (CB02011-1); 3. Cheongju, CB, (CB02054-1); 4. Buyoung, CB, (CB02058-1); 5. Jeungpyeong. CB, (CB02064-1); 6. Goesan, CB, (CB02071, CB02073, CB02073-1); 7. Mt. Geryong, Chung-nam (CN), (CN02018-1); 8. Gooryong, CN (CN02023-1); 9. Deoksan, CN, (CN02028-1); 10. Manripo, CN (CN02035-1); 11. Booyeo, CN, (CN02036-1, CN02037-1); 12. Cheonan, CN, (CN02042-1); 13. Choonjangdae, CN, (CN02046-1); 14. Mt. Deukyou, Jeon-buk (JB), (JB02010-1); 15. Byunsan, JB, (JB02018-1); 16. Yeosu, Jeon-nam (JN), (JN02005-1, JN02010-1); 17. Nogodan, JN, (JN02020-1); 18. Chilgok, Gyeong-buk (KB), (KB02005-1, KB02006-1); 19. Mt. Palgong, KB, (KB02011-1, KB02011-2); 20. Keumsan, GyeongGi (KK), (KK02005-1); 21. Gwangju, KK (KK02006-1); 22. Changnyoung, Gyeong-nam (KN), (KN02003-1, KN02005-1, KN02018-1); 23. Youngwol, Gang-won (KW), (KW02006-1, KW02009-1, KW02010-1, KW02014-1); 24. Udo, Jeju (JJ), (JJ02001); 25. Bija forest, JJ, (JJ02002).

TTG GTT TCC-3') for 30 cycles (1 cycle at 95°C for 3 min, at 47°C for 1 min, at 72°C for 1 min; 28 cycles at 95°C for 30 sec, at 47°C for 1 min at 72°C for 1 min; 1 cycle at 95°C for 30 sec, at 47°C for 1 min, at 72°C for 10 min). Second PCR products were sequenced on automatic sequencer ABIPRISM™ (Perkin-Elmer, USA at Eugenetech Co., Korea).

The most similar sequences for each sequence from AM fungal spores were obtained from the GenBank through BLAST at the National Center for Biotechnology Information (NCBI) and used for molecular identification of each AM fungal spores. Clustal X (Thompson *et al.*, 1994) was used for alignment of DNA sequence and phylogenetic analysis using the sequences from the AM fungal spores using neighbor-joining methods with bootstrap (Saitou and Nei, 1987). *Endogone pisiformis* was used as an outgroup.

Results and Discussion

Morphological identification. Spores isolated from soils were separated and identified, if possible, using morphological characters including spore size, color, wall structures and reactions with Melzer's reagent (Fig. 2). A total of 15 morpho-species in 7 genera were identified; 1 species in *Acaulospora*, 1 species in *Archaeospora*,

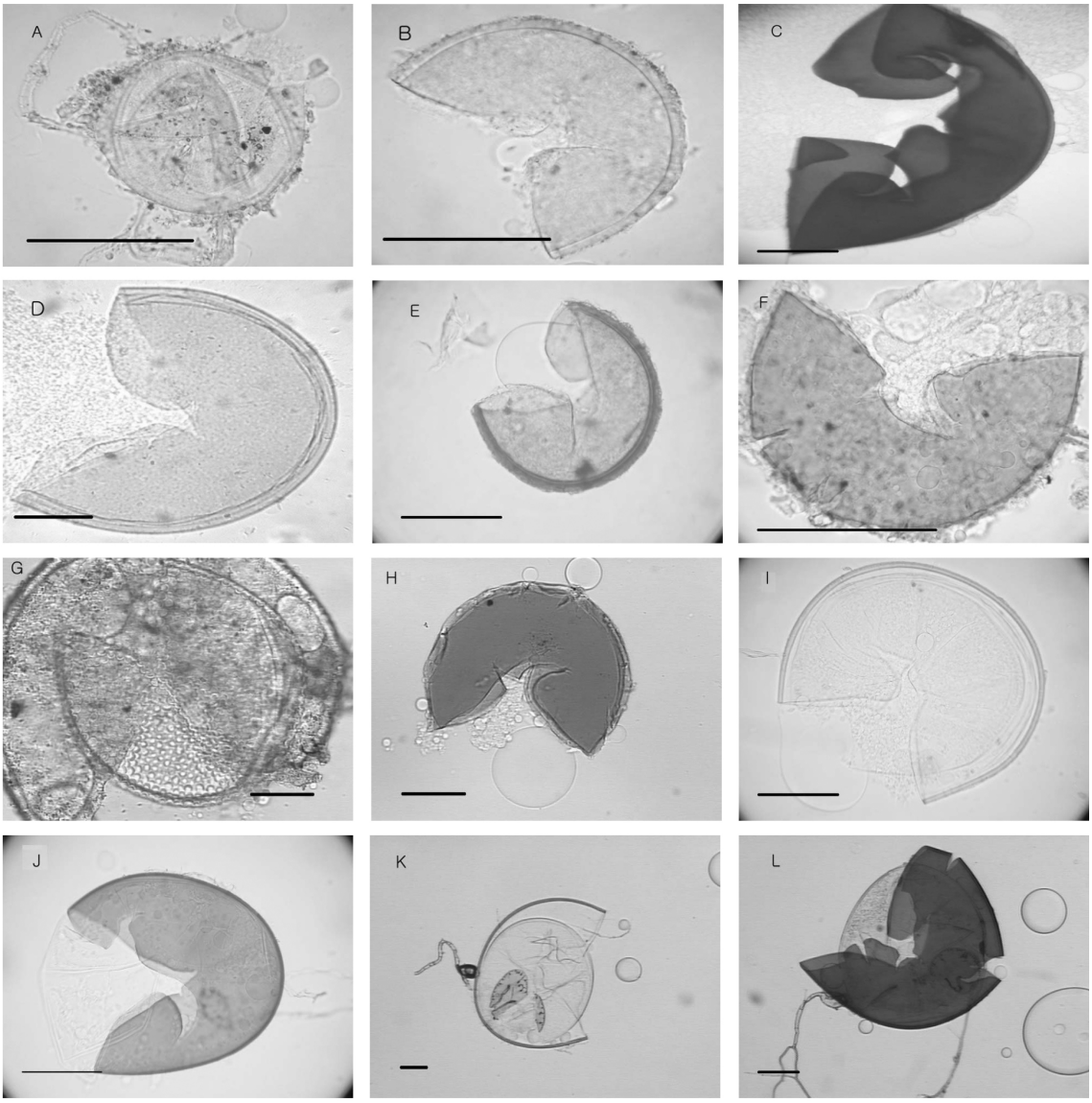

Fig. 2. Spores of arbuscular mycorrhizal fungi used in this study. A. *Paraglomus occultum* (KW02009-1), B. *Glomus* sp. yellow2 (KB02011-1), C. *Gigaspora* sp. A (JN02010-1), D. *Gigaspora gigantea* (CN02023-1), E. *Glomus etunicatum* (KK02006-1), F. *Glomus etunicatum* (CN02046-1), G. *Archaeospora leptoticha* (KW02006-1), H. *Glomus* sp. yellow1 (CB02071), I. *Scutellospora weresubiae* (CN02037-1), J. *Scutellospora castanea* (CB01006-1), K. *Scutellospora cerradensis* (CB02058-1) and L. *Scutellospora cerradensis* (CB02058-1) stained with Melzer's reagent, Scale bar = 50 μm.

6 species in *Glomus*, 2 species in *Gigaspora*, 1 species in *Paraglomus* and 4 species in *Scutellospora.*

Molecular identification. AM fungal spores were grouped based on spore morphology and representative spores from each group were selected. Total 36 sequences were obtained from the spores and were compared with GenBank database for molecular identification. The most similar sequences were obtained from the sequence database in NCBI using BLAST and the sequences were grouped into 13 species in 6 genera (Table 1). In this grouping, several sequences were not consistent with morphological identification. This result was mainly due to insufficient AM fungal sequence data in the database for sequences from this study as well as limitation in the identification using morphological characteristics of spores.

These 36 sequences from AM fungal spores were phylogenetically analyzed with other 24 sequences from NCBI data. The phylogram obtained from the analysis grouped

Table 1. Consensus species from morphological and molecular identification of arbuscular mycorrhizal fungal spores

Isolates	The most similar sequences in GenBank database		Molecular identification[1]	Consensus species from morphological and molecular identification
	Species (Accession number)	Sequence similarity (%)		
	Archaeospora			
KW02006-1	*Ar. leptoticha* (AB047306)	708/720 (98%)	*Ar.* sp. A	*Ar. leptoticha*
	Paraglomus			
KWO2009-1	*P. occultum* (AJ276081)	659/734 (94%)	*P.* sp. A	*P. occultum*
CB02064-1	*P. occultum* (AJ276081)	743/751 (98%)	*P.* sp. B	*P. occultum*
	Glomus			
CN02018-1	*G. mosseae* (AJ306438)	748/755 (99%)	*G.* sp. A	*G. mosseae*
KK02005-1	*G. mosseae* (AJ306438)	748/755 (99%)	*G.* sp. A	*G. mosseae*
JN02005-1	*G. mosseae* (AJ306438)	753/757 (99%)	*G.* sp. A	*G. mosseae*
CB02073-1	*G. mosseae* (AJ306438)	757/762 (99%)	*G.* sp. A	*G. mosseae*
KN02003-1	*G. mosseae* (AJ306438)	749/754 (99%)	*G.* sp. A	*G. mosseae*
CN02036-1	*G. mosseae* (AJ306438)	734/738 (99%)	*G.* sp. A	*G. mosseae*
KW02014-1	*G. mosseae* (AJ306438)	743/750 (99%)	*G.* sp. A	*G. mosseae*
JN02020-1	*G. mosseae* (AJ306438)	635/650 (97%)	*G.* sp. A	*G. mosseae*
JJ02001	*G. clarum* (Y17648)	698/719 (97%)	*G.* sp. B	*G. clarum*
CB02071	*G. claroideum* (AJ276075)	663/714 (92%)	*G.* sp. C	*G.* sp. yellow1
KB02011-1	*G. lamellosum* (AJ276087)	680/692 (98%)	*G.* sp. D	*G.* sp. yellow2
CB02073	*G. etunicatum* (Y17639)	756/759 (99%)	*G.* sp. E	*G. etunicatum*
KN02018-1	*G. etunicatum* (Y17639)	681/689 (99%)	*G.* sp. E	*G. etunicatum*
KK02006-1	*G. claroideum* (AJ276080)	756/759 (98%)	*G.* sp. E	*G. etunicatum*
CB02011-1	*G. lamellosum* (AJ276087)	718/738 (97%)	*G.* sp. E	*G. etunicatum*
KN02005-1	*G. lamellosum* (AJ276087)	758/759 (99%)	*G.* sp. E	*G. etunicatum*
CN02046-1	*G. lamellosum* (AJ276087)	756/759 (99%)	*G.* sp. E	*G. etunicatum*
	Diversispora			
KW02010-1	*G. etunicatum* (Y17644)	759/763 (99%)	*D.* sp. A.	*G. etunicatum*
	Acaulospora			
JB02010-1	*Ac. longula* (AJ306439)	703/711 (98%)	*A.* sp. A	*A. longula*
KB02006-1	*Ac. longula* (AJ306439)	753/762 (98%)	*A.* sp. A	*A. longula*
CN02035-1	*Ac. longula* (AJ306439)	747/751 (99%)	*A.* sp. A	*A. longula*
	Gigaspora			
JN02010-1	*Gi. gigantea* (Z14010)	739/744 (99%)	*Gi.* sp. A	*Gi.* sp.
CN02023-1	*Gi. gigantea* (Z14010)	750/753 (99%)	*Gi.* sp. B.	*Gi. gigantea*
CN02028-1	*Gi. gigantea* (Z14010)	726/735 (98%)	*Gi.* sp. B	*Gi. gigantea*
KB02011-2	*Gi. gigantea* (Z14010)	722/749 (96%)	*Gi.* sp. B	*Gi. gigantea*
KB02005-1	*Gi. gigantea* (Z14010)	707/721 (98%)	*Gi.* sp. B	*Gi. gigantea*
JB02018-1	*Gi. gigantea* (Z14010)	644/648 (99%)	*Gi.* sp. B	*Gi. gigantea*
	Scutellospora			
JJ02002	*Sc. aurigloba* (AJ276093)	621/630 (98%)	*S.* sp. A	*S. aurigloba*
CN02037-1	*Sc. weresubiae* (AJ306444)	701/724 (96%)	*S.* sp. B	*S. weresubiae*
CN02042-1	*Sc. weresubiae* (AJ306444)	679/684 (99%)	*S.* sp. B	*S. weresubiae*
CB01006-1	*Sc. castanea* (AF038590)	739/751 (98%)	*S.* sp. C	*S. castanea*
CB02058-1	*Sc. cerradensis* (AB041344)	715/722 (99%)	*S.* sp. D.	*S. cerradensis*
CB02054-1	*Sc. cerradensis* (AB041344)	716/724 (98%)	*S.* sp. D	*S. cerradensis*

[1]See Fig. 3.

Fig. 3. Neighbor-joining consensus phylogram for partial 18S rDNA sequence of arbuscular mycorrhizal fungal spores. Bootstrapping, 1000 replicates. Scale bar = 10% sequence divergence.

the 36 sequences in to 16 clades (Fig. 3). Using morphological and molecular grouping, the clades were grouped into 15 groups (Table 1). Most of clades in the phylogram were consistent with morphological identification; *Archaeospora* sp. A, *Paraglomus* sp. B, *Glomus* sp. A, *Glomus* sp. E, *Acaulospora* sp. A, *Glomus* sp. F, *Scutellospora* sp. A, *Scutellospora* sp. B, *Scutellospora* sp. C, *Scutellospora* sp. D and *Gigaspora* sp. B.

Two species were not identified in genus *Glomus*. Spores of *Glomus* sp. C (Fig. 2H) was not able to be identified using morphological characteristics and described as *Glomus* sp. yellow1. Although the most similar sequence with this spore was *Glomus claroideum*, these two sequences were positioned on a quit distance clade in the phylogram. Thus, this species remains as unidentified species, *Glomus* sp. yellow1. *Glomus* sp. D (Fig. 2B) was unidentified morphologically and the most similar sequence was *G. lamellosum*. In the phylogenetic tree (Fig. 3), this species was in a same clade with *G. lamellosum* (AJ27687), *G. claroideum* (AJ276075) and *G.* sp. E (Fig.

2F). The spore color and wall structure were difficult to separate with *G. etunicatum* and this species also remain unidentified species as described as *G.* sp. yellow2.

In *Paraglomus* sp. A (Fig. 2A), spore size and wall structure were similar with *P. occultum* (= *Glomus occultum*), but *P.* sp. A was grouped into a different clade from *P.* sp. B and *P. occultum*. *P.* sp. A might be same species as *P. occultum* or close species. This genus was a new genus separated from *Glomus*, consisting two species forming indistinguishable spores from those of *Glomus* species (Morton and Redecker, 2001). Spore isolate KK02006-1 (Fig. 2E) was identified as *G. etunicatum* in morphological characters. *G. claroideum* has 4 wall layers and it is different from *G. etunicatum* which has only 1 or 2 wall layers (Becker and Gerdemann, 1977). Although the most similar sequences was *G. claroideum*, it was decided as *G. etunicatum* using information from phylogenetic analysis which the sequence from this spore was within the clade of *G. etunicatum* in the phylogram, as well as morphological characters. Also, the sequences of CN02046-1 (Fig. 2F), CB02011-1 and KN02005-1 were close to sequence of *G. lamellosum* (AJ276087), but spore isolates CN02046-1, CB02011-1 and KN02005-1 with two spore wall were different from *G. lamellosum* (Dalpe *et al.*, 1992) with tree wall layers and formed a same clade in the phylogenetic tree. These spores in this clade were identified as *G. etunicatum* (Fig. 3).

These molecular analyses of AM fungal spores using blast and phylogenetic tree showed mostly consistent result with morphological identification except for one isolate KW02010. This species was morphologically identified as *G. etunicatum* and the sequence was also similar with the species. However, phylogenetic analysis showed highly divergent from the clade of *G. etunicatum* (Fig. 3). A group of *G. etunicatum* was formed a clade within *Glomus* group B (GlGrB) and the other group with isolate KW02010 was in a new genus *Diversispora*, which was separated from *Glomus* using molecular data and produce indistinguishable spores with *Glomu*s (Walker and Schuessler, 2004).

The isolate *Gigaspora* sp A (Fig. 2C) was identified as *Gi. gigantea* from sequence similarity. However, phylogenetic relationship showed close relationship with *Gi. albida* (Z14009). The spores of *Gi. albida* are often confused with *Gi. gigantea* with similar color, but the spores used in this study, have 25 μm thick wall which was thicker than that of *Gi. gigantea* (Fig. 2D). This spore remained as an unidentified *Gigaspora* species.

Out of 15 AM fungal species analyzed in this study, 12 species were identified and 3 species remained as unidentified. The 4 species of 12 identified species was not reported in Korea; *Archaeospora leptoticha* (Schenck & Smith) Morton & Redecker (Fig. 2G), *Scutellospora castanea* Walker (Fig. 2J), *Scutellospora cerradensis* Spain & Miranda (Fig. 2K), *Scutellospora weresubiae* Koske & Walker (Fig. 2I).

With morphological identification, this study identified AM fungal spores based on partial 18S rDNA of the spores. We used a primer pair AML1/AML2 specific to AM fungi and these primers amplified DNA from all of the species used in this study, suggesting that the primers are reliable for use in identifying AM fungal hyphae in the roots. Also, the sequence data of AM fungal spores from this study could be use for identify AM fungi colonizing roots. Schuessler *et al.* (2001) also grouped AM fungi into a new phylum Glomeromycota using sequences of small subunit of ribosomal RNA. To give a more reliable phylogenetic tree, several genes as well as other characteristics such as morphological and biochemical characters have to be used (Rokas *et al.*, 2003).

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